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Ability of Adeno-Associated Virus Serotype 8-Mediated Hepatic Expression of Acid α-Glucosidase to Correct the Biochemical and Motor Function Deficits of Presymptomatic and Symptomatic Pompe Mice

Robin J. Ziegler, Scott D. Bercury, Jonathan Fidler, Michael A. Zhao, Joseph Foley, Tatyana V. Taksir, Susan Ryan, Bradley L. Hodges, Ronald K. Scheule, Lamya S. Shihabuddin, and Seng H. Cheng

Abstract

The availability of a murine model of Pompe disease has enabled an evaluation of the relative merits of various therapeutic paradigms, including gene therapy. We report here that administration of a recombinant adeno-associated virus serotype 8 (AAV8) vector (AAV8/DC190-GAA) encoding human acid α-glucosidase (GAA) into presymptomatic Pompe mice resulted in nearly complete correction of the lysosomal storage of glycogen in all the affected muscles. A relatively high dose of AAV8/DC190-GAA was necessary to attain a threshold level of GAA for inducing immunotolerance to the expressed enzyme and for correction of muscle function, coordination, and strength. Administration of AAV8/DC190-GAA into older Pompe mice with overt disease manifestations was also effective at correcting the lysosomal storage abnormality. However, these older mice exhibited only marginal improvements in motor function and no improvement in muscle strength. Examination of histologic sections showed evidence of skeletal muscle degeneration and fibrosis in aged Pompe mice whose symptoms were abated or rescued by early but not late treatment with AAV8/DC190-GAA. These results suggest that AAV8-mediated hepatic expression of GAA was effective at addressing the biochemical and functional deficits in Pompe mice. However, early therapeutic intervention is required to maintain significant muscle function and should be an important consideration in the management and treatment of Pompe disease.

Introduction

POMPE DISEASE is a rare metabolic myopathy caused by a deficiency of the lysosomal hydrolase α-glucosidase (GAA). Consequently, affected subjects accumulate progressively higher amounts of undegraded glycogen in their lysosomes and autophagosomes, leading to distension of the organelles and subsequent cellular and tissue dysfunction (Hirschhorn and Reuser, 2001; Fukuda *et al.*, 2006). Depending on the extent of enzyme deficiency, Pompe patients present with a continuum of clinical severity, with the respiratory, cardiac, and skeletal muscles being the most seriously affected (Vorgerd *et al.*, 1998; van den Hout *et al.*, 2003). Several therapeutic modalities including the use of gene, protein, and small-molecule therapies are currently under active consideration for treating this and other related lysosomal storage disorders (Beck, 2007; Koeberl *et al.*, 2007; Okumiya *et al.*, 2007). Of these, only enzyme replacement therapy with recombinant GAA has been approved for treating Pompe disease in infants.

Preclinical studies in a murine model of Pompe disease (Raben *et al.*, 1998) have shown that enzyme replacement therapy with high doses (20–100 mg/kg) of recombinant GAA is effective at reversing the glycogen storage abnormality in the affected tissues (Kikuchi *et al.*, 1998; Bijvoet *et al.*, 1999; Raben *et al.*, 2002, 2003). However, the skeletal muscles, particularly of older animals, appeared to be somewhat more refractory to treatment. Incomplete correction of the accumulated substrate in skeletal muscle was noted even when the animals were repeatedly administered high doses of the enzyme. The efficiency of substrate clearance from the different muscle groups was variable, with the type II muscle fibers being more resistant to treatment than the type I muscle fibers (Fukuda *et al.*, 2006; Raben *et al.*, 2006). It has been suggested that this decreased efficacy in type II fibers might be due to lower amounts of the receptor (cation-in-

Genzyme, Framingham, MA 01701.

dependent mannose 6-phosphate receptor) that is primarily responsible for cellular uptake of the enzyme. Data also suggested that type II fibers might have higher autophagic activity that could account for the increase in autophagosomes in these cells (Fukuda *et al.*, 2006). Subsequent clinical studies with enzyme therapy in infantile Pompe subjects showed a similar profile of greater clearance of glycogen from the heart than skeletal muscles (Kishnani *et al.*, 2006, 2007). Analysis of muscle biopsies also showed a correlation between subjects with limited or minimal motor response and those harboring a greater proportion of type II fibers (Thurberg *et al.*, 2006). Hence, strategies that could enhance delivery of the enzyme to these muscle types might improve clinical outcome. One proposed approach involves modifying the carbohydrate moieties on GAA to improve its affinity for the cation-independent mannose 6-phosphate receptor (Zhu *et al.*, 2005).

Gene augmentation therapy using recombinant adenoviral or adeno-associated viral (AAV) vectors encoding GAA represents an alternative strategy to enzyme therapy (Fraites *et al.*, 2002; Sun *et al.*, 2003, 2005; Xu *et al.*, 2005; Mah *et al.*, 2007). It has been shown that significant clearance of glycogen deposits from all the affected muscles, including those of older Pompe mice, could be attained after systemic delivery of the recombinant viral vectors encoding GAA. The extent and rate of clearance of the substrate after gene transfer appeared to be more effective than were achieved by intermittent delivery of the recombinant enzyme, at least when tested in Pompe mice. This result was likely due to the ability of the viral vectors to confer sustained and high-level production of GAA over the test period. Gene therapy also offered the additional potential benefit of conferring immunotolerance to the expressed transgene product (Ziegler *et al.*, 2004, 2007; Franco *et al.*, 2005), an important consideration in the treatment of genetic diseases such as Pompe disease. However, despite the encouraging data from these earlier studies, it was not clear whether clearance of tissue glycogen per se necessarily translated to a corresponding improvement in motor function.

Here, the relationship between a reduction in lysosomal storage of glycogen and improvement in motor function, particularly in the context of differing extents of preexisting tissue pathology, was examined. Presymptomatic and symptomatic Pompe mice with more advanced tissue pathology were administered a recombinant AAV8 vector encoding GAA and the effects of treatment at correcting the biochemical and functional deficits examined. We showed, as observed previously with enzyme replacement therapy, that relatively high levels of expression of GAA were necessary for efficacy. Complete clearance of tissue glycogen and the induction of immunotolerance to GAA were realized only in Pompe mice administered high doses of the recombinant viral vector. It would also appear that preexisting muscle pathology was a confounding factor that influenced the extent of recovery in motor function. Together, these results highlight considerations for therapy of Pompe disease.

Materials and Methods

Generation of AAV8/DC190-GAA

Appropriate primers were used to amplify a 2.8-kb fragment of the cDNA for human GAA from pAAV-CBGAApA (Sun *et al.*, 2003) that was then subcloned into the Topo vector (Invitrogen, Carlsbad, CA) to generate pTopo-GAA. pDC190-GAA was generated by ligating the 2913-bp *SpeI–XbaI* GAA fragment from pTopo-GAA into the *BamHI–XbaI* sites in pDC190-agal (Ziegler *et al.*, 2004). This placed the expression of GAA under the transcriptional control of the human serum albumin promoter (DC190). The expression cassette was then subcloned into the *BstBI–AvrII* sites of the AAV2 previral plasmid pAAVSP70. Recombinant AAV8 serotype vectors encoding GAA (AAV8/DC190-GAA) were generated by the standard triple plasmid transfection method and purified by cesium chloride ultracentrifugation (Gene Therapy Vector Core, University of Pennsylvania, Philadelphia, PA). The titer of the virus was determined by a real-time TaqMan polymerase chain reaction (PCR) assay (ABI PRISM 7700; Applied Biosystems, Foster City, CA) with primers that were specific for the bovine growth hormone polyadenylation signal sequence.

Animal studies

The Pompe mice used in these studies have a targeted disruption of exon 6 in the gene for GAA and consequently lack enzymatic activity (Raben *et al.*, 1998). Their phenotype resembles the rapidly progressive disease observed in Pompe infants. Pompe and wild-type B6129SF2/J mice (Jackson Laboratory, Bar Harbor, ME) were housed in small groups with free access to food and water in a facility operated under the guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD). All study protocols were reviewed and approved by the institutional animal care and use committee. Gene delivery was achieved by intravenous administration of 0.2 ml of the recombinant viral vector into a tail vein of the mice. Blood samples were collected from the orbital venous plexus under anesthesia (2–3% isoflurane), using heparinized microhematocrit capillary tubes, and after serum separation were stored at –80°C. At various time points mice were removed from the housing area to a separate room and subjected to a battery of functional tests. At the end of the studies mice were killed by intraperitoneal injection of Euthasol (pentobarbital sodium and phenytoin sodium; Virbac, Fort Worth, TX). The animals were then perfused with phosphate-buffered saline, using a peristaltic pump attached to a 25-gauge needle inserted into the left ventricle of the heart. After perfusion, various organs were harvested and either processed with fixative or snap-frozen on dry ice and stored at –80°C until they were ready for further analysis.

Methods for measuring tissue and serum levels of GAA

Frozen tissues were weighed and then homogenized in sterile water (at a concentration of approximately 100 mg/ml), using a Tissue-Tearor (Biospec Products, Bartlesville, OK). The homogenates were sonicated for 15 sec with a VirSonic 100 sonicator (VirTis, Gardiner, NY), and then centrifuged at 15,000 rpm on a microcentrifuge for 15 min at 4°C to remove cellular debris. Tissue and serum levels of GAA were determined by either an enzyme-linked immunosorbent assay (ELISA) or enzymatic activity assay.

For the ELISA, 96-well high-protein-binding microtiter plates (Corning/Costar, Costar, NY) were first coated with

a monoclonal antibody specific for human GAA (Genzyme, Cambridge, MA). The wells were then treated with blocking solution (Tris-buffered saline containing 5% non-fat milk [Bio-Rad, Hercules, CA] and 0.05% Tween 20, pH 7.5) for 1 hr at 4°C. Serum and tissue samples were diluted in blocking buffer, loaded in duplicate wells, and incubated for 1 hr at 37°C. Plates were then treated consecutively with a secondary biotinylated monoclonal antibody and streptavidin-horseradish peroxidase (2 μg/ml; Pierce Biotechnology, Rockford, IL). After washing, the plates were developed by the addition of 3,3′,5,5′-tetramethylbenzidine (BioFX Laboratories, Owings Mills, MD) and the absorbance at 450 nm was measured with a Spectromax Plus plate reader (Molecular Devices, Sunnyvale, CA).

GAA enzymatic activity was measured with the fluorogenic substrate 4-methylumbelliferyl α-D-glucopyranoside (Sigma-Aldrich, St. Louis, MO). Lysates were diluted in a buffer containing 0.2 *M* sodium acetate, 0.4 *M* potassium chloride, and 0.01% bovine serum albumin (BSA), pH 3.9, and loaded into duplicate wells of 96-well microtiter plates. The fluorogenic substrate was added to a final concentration of 1.3 mg/ml and the reaction mixture was incubated at 37°C for 2 hr. The reaction was stopped by the addition of 0.5 *M* sodium bicarbonate buffer, pH 10.65, and the fluorescence at 450 nm was measured with a Spectromax Gemini plate reader (Molecular Devices). For both the ELISA and activity assays, GAA levels were extrapolated from a standard curve made on the basis of purified recombinant human acid α-glucosidase (Genzyme). The specific activity of the virus-derived GAA was determined to be similar to that of the recombinant enzyme purified from Chinese hamster ovary cells.

Measurement of glycogen levels

To measure tissue glycogen levels, lysates were first digested with α-amyloglucosidase (0.54 mg/ml; Sigma-Aldrich) in 25 m*M* potassium acetate buffer, pH 5.5. The released glucose in the test samples and the levels of endogenous glucose from undigested samples were quantitated with an Amplex red glucose/glucose oxidase assay kit (Invitrogen) according to the manufacturer's instructions. Glycogen levels were determined by subtracting the glucose levels in the undigested samples from those in the digested samples. Bovine liver glycogen (Sigma-Aldrich) was used as a reference standard.

Antibody titer determinations

Levels of GAA antibodies in serum were determined by ELISA. Serial dilutions of serum were added to wells of 96-well plates coated with recombinant human α-glucosidase (5 μg/ml). Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG), IgM, and IgA (Invitrogen Zymed, South San Francisco, CA). After washing, the plates were developed by the addition of 3,3′,5,5′ tetramethylbenzidine (BioFX Laboratories) and the absorbance at 450 nm was measured with a Spectromax Plus plate reader (Molecular Devices). Titers were defined as the reciprocal of the highest dilution of serum that produced an OD_{450} greater than or equal to 0.1.

Immunotolerance studies

To determine whether mice had developed immunotolerance to the expressed GAA, 50 μg of purified recombinant human α-glucosidase was emulsified with Freund's complete adjuvant (1:1 ratio) and injected intraperitoneally. In some studies, mice were subjected to a second challenge with antigen after emulsification in Freund's incomplete adjuvant (1:1 ratio). Blood was collected at various time points and assayed for the presence of anti-GAA antibodies, using the ELISA described above.

Functional tests

To avoid possible variations associated with circadian rhythm, all functional tests were performed between 1:00 and 4:00 P.M. Muscle coordination was assessed with the accelerating RotaRod in combination with the Smartrod program (AccuScan Instruments, Columbus, OH). Mice were placed on a 30-mm-diameter cylinder that was elevated approximately 18 in. above a cushioned base. The speed of the cylinder rotation was programmed to increase with constant acceleration from stationary to a final speed of 30 rpm over the course of 60 sec. Latency to fall from the rod was recorded automatically with a light beam sensor. Each animal was subjected to three trials with a 5-min resting period between each trial and the average latency was determined.

Mice were tested for muscle strength by the wire hang assay. Animals were placed onto a wire screen that was then inverted above a cushioned pad (~13 in. above pad) so that they were suspended by their limbs. The latency of the mice to fall was recorded visually with a stopwatch. Each animal was subjected to two trials at any time point and the average latency was recorded. Statistical analysis was performed by analysis of variance (ANOVA) multivariable software (GraphPad Software, San Diego, CA).

Histopathology

Mouse quadriceps were fixed in 10% neutral buffered formalin (Electron Microscopy Sciences, Hatfield, PA) and embedded in paraffin. Sections were cut at a thickness 5 μm for hematoxylin–eosin staining and at a thickness of 7 μm for Masson's trichrome staining. Spinal column sections were also fixed in 10% neutral buffered formalin and were then decalcified in TBD-2 solution (Thermo Shandon, Pittsburgh, PA) and embedded in paraffin. Sections cut at a thickness of 5 μm were prepared and stained with hematoxylin–eosin. All pictures taken were of representative sections at an original magnification of ×40, using an Olympus AX70 microscope (Olympus, Center Valley, PA) equipped with an Optronics camera and PictureFrame digital imaging software (Optronics, Goleta, CA).

Results

Efficacy of AAV8/DC190-GAA in correcting glycogen storage in affected muscles of Pompe mice is dose dependent

Previous studies using purified recombinant acid α-glucosidase (GAA) in Pompe mice had indicated that relatively high doses of the enzyme are necessary to reduce the bur-

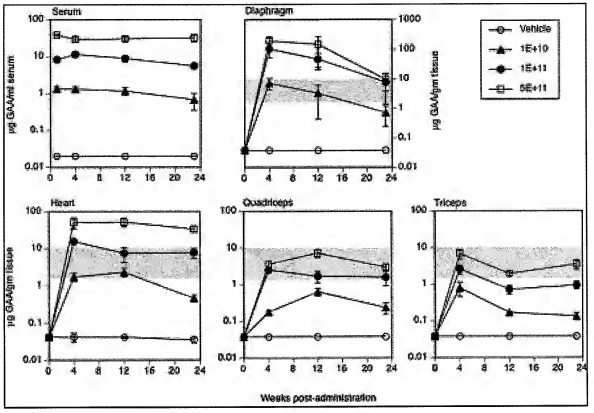
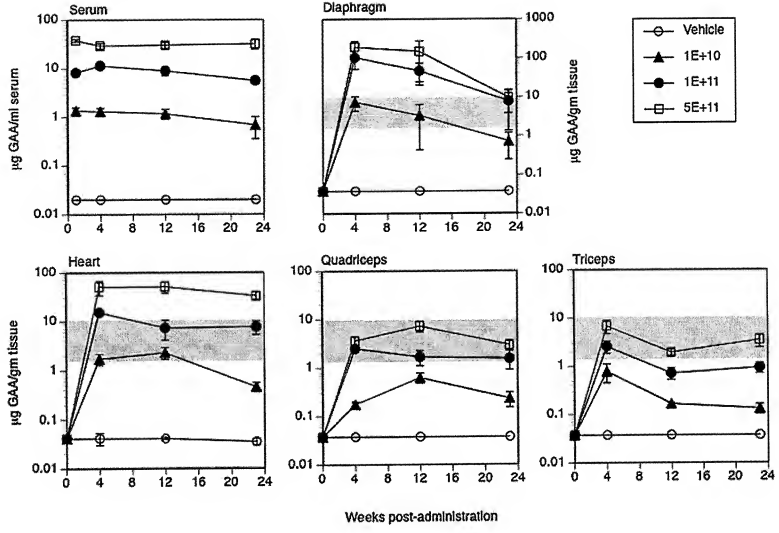


FIG. 1. Tissue levels of GAA after systemic administration of AAV8/DC190-GAA to Pompe mice. Cohorts of 12-week-old male Pompe mice were administered vehicle or AAV8/DC190-GAA at 10^{10}, 10^{11}, or 5×10^{11} DRP via the tail vein. Animals were killed 4, 12, and 23 weeks posttreatment and their organs were analyzed for levels of GAA, using an ELISA. Sera were also collected by retro-orbital bleeding at the times indicated and analyzed for enzyme levels. The shaded area within the graph represents the range of GAA levels observed in normal (C57BL/6) mouse tissues. Data are expressed as means ± SEM (n = 5 animals per group).

den of glycogen storage, particularly in the skeletal muscles (Raben *et al.*, 2003). Consequently, a recombinant AAV8 serotype vector that demonstrates highly efficient hepatic transduction (Gao *et al.*, 2002) was developed and its activity was evaluated in Pompe mice. Cohorts of approximately 12-week-old Pompe mice were administered 10^{10}, 10^{11}, or 5×10^{11} DNase-resistant particles (DRP) of a recombinant AAV8 vector encoding human GAA (AAV8/DC190-GAA) under the transcriptional control of the liver-restricted promoter DC190 (Ziegler *et al.*, 2004). A dose-dependent increase in hepatic transduction was realized as evidenced by the correspondingly higher levels of enzyme secreted into the circulation (Fig. 1). These serum levels translated into progressively higher levels of uptake of the enzyme into the various muscles assayed. Circulating and tissue levels of GAA were sustained for the 23-week duration of the study. An exception was the diaphragm, where GAA levels decreased approximately 10-fold toward the end of the study (Fig. 1). The levels of hydrolase attained in the tissues of mice treated with the higher doses (10^{11} and 5×10^{11} DRP/mouse) of the viral vector were either within the normal range observed in wild-type mice or supraphysiological. Animals administered the lowest dose (10^{10} DRP/mouse) of AAV8/DC190-GAA exhibited nearly normal levels of GAA in the diaphragm and heart but an approximately 5- to 10-fold lower level than normal in the quadriceps and triceps (Fig. 1).

Analysis of glycogen levels in the tissues of treated Pompe mice showed they were significantly decreased when compared with vehicle-treated, age-matched controls (Fig. 2). The extent of correction of the storage abnormality was commensurate with the dose of AAV8/DC190-GAA administered, that is, the greatest reduction in glycogen was noted in mice that received the highest dose of viral vector and the least reduction was seen in the cohort that received the lowest dose. Moreover, the rates at which glycogen was cleared reflected the levels of GAA attained in the corresponding tissues, that is, more rapid clearance was observed in mice that had attained higher levels of the enzyme. Glycogen in Pompe mice administered the highest dose of AAV8/DC190-GAA (5×10^{11} DRP/mouse) was reduced to near-basal levels at 23 weeks posttreatment. At the lower doses, clearance was incomplete, particularly in the quadriceps and triceps, suggesting that these muscles were more refractory to treatment than the heart and diaphragm (Fig. 2). On the basis of these results, a dose of 10^{11} DRP of AAV8/DC190-GAA per mouse would appear to be minimally required to address the aberrant lysosomal accumulation of glycogen in Pompe mice of this age.


Serum
Diaphragm
Heart
Quadriceps
Triceps
µg GAA/ml serum
µg GAA/gm tissue
µg GAA/gm tissue
Weeks post-administration
Vehicle
1E+10
1E+11
5E+11

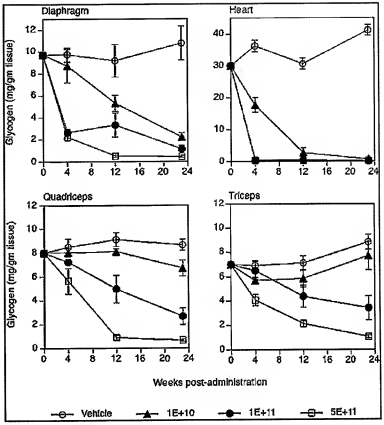


FIG. 2. Efficacy of administration of AAV8/DC190-GAA to Pompe mice. Tissues obtained from the study outlined in the caption to Fig. 1 were processed and assayed for glycogen levels, using the Amplex Red glucose assay. Data are expressed as means ± SEM ($n = 5$ animals per group).

A high threshold level of expressed GAA is necessary to induce immunotolerance to the neo-antigen

Previously, it has been reported that intravenous administration of recombinant AAV vectors encoding various lysosomal enzymes under the transcriptional control of a liver-restricted promoter conferred immunotolerance to the expressed enzyme (Ziegler *et al.*, 2004, 2007; Barbon *et al.*, 2005; McEachern *et al.*, 2006). Temporal acquisition of immunotolerance to the enzyme was inversely related to the level of expression attained, with higher levels supporting a more rapid induction of the immunotolerized state. We also asked whether this phenomenon applied to AAV8-mediated hepatic expression of GAA in the Pompe mice. As in the previous studies, groups of 12-week-old Pompe mice were injected via the tail vein with three different doses (10^{10}, 10^{11}, and 5×10^{11} DRP) of AAV8/DC190-GAA. No antibodies against human GAA were detected in the serum of mice administered 5×10^{11} DRP of AAV8/DC190-GAA at 8 weeks postinjection (Fig. 3A). Immunologic challenge of these mice at the 8-week time point with 50 μg of purified recombinant GAA in Freund's complete adjuvant did not elicit a humoral immune response to the antigen. A subsequent challenge of these animals at the 16-week time point was also uneventful. In contrast, identical immunologic challenges of vehicle-treated mice at the same time points generated rapid and robust levels of antibodies against the expressed enzyme (Fig. 3A). Hence, as shown for the other lysosomal hydrolases, hepatically mediated expression of high levels of GAA conferred immunotolerance to the human enzyme. This effect was also observed in Pompe mice administered 10^{11} DRP but not the lower amount of 10^{10} DRP of AAV8/DC190-GAA per mouse (Fig. 3B). Antigenic challenge of the animals administered 10^{11} DRP of virus at the 12-week time point did not elicit production of antibodies. Interestingly, mice treated with the lowest dose (10^{10} DRP) of AAV8/DC190-GAA generated antibodies against GAA even before the immunologic challenge with recombinant protein in Freund's complete adjuvant. Antibody titers to human GAA were detected soon after administration of AAV8/DC190-GAA, continued to rise over time, and were further boosted by an immune challenge at the 12-week time point (Fig. 3B). Together, these data suggest that acquisition of immunotolerance to the expressed GAA is directly correlated with the level of enzyme generated.

Correction of muscle coordination and strength is correlated with the extent of glycogen clearance in presymptomatic Pompe mice

The effect of expressing GAA and the subsequent clearing of the offending substrate from the tissues of Pompe mice on motor function was also examined. Approximately 8-week-old Pompe mice were administered vehicle or AAV8/DC190-GAA at 10^{11} or 5×10^{11} DRP per mouse. At this age, the mice have measurable accumulation of glycogen but do not exhibit any measurable motor function or behavioral deficits. Animals were killed 13 months posttreatment and the tissues were analyzed for the levels of GAA and glycogen (Fig. 4). All tissues from the AAV8/DC190-

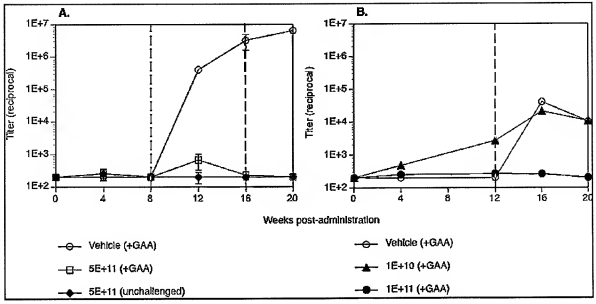


FIG. 3. Titers of antibodies to human GAA in Pompe mice. Groups of 12-week-old male Pompe mice were injected intravenously with vehicle or with AAV8/DC190-GAA at 10^{10}, 10^{11}, or 5×10^{11} DRP. Mice were bled before treatment and subsequently at 4, 8, 12, 16, and 20 weeks posttreatment. (A) Mice administered vehicle or 5×10^{11} DRP of AAV8/DC190-GAA were challenged 8 weeks posttreatment by a single intraperitoneal injection of 50 μg of purified recombinant GAA emulsified in Freund's complete adjuvant. The animals were rechallenged at 16 weeks posttreatment with 50 μg of purified recombinant GAA emulsified in Freund's incomplete adjuvant. As a control, one cohort of mice that had been treated with 5×10^{11} DRP of AAV8/DC190-GAA was unchallenged. (B) Mice administered vehicle or AAV8/DC190-GAA (10^{10} or 10^{11} DRP) were challenged 12 weeks posttreatment by a single intraperitoneal injection of 50 μg of purified recombinant GAA emulsified in Freund's complete adjuvant. Vertical dotted lines refer to the timing of the immunological challenges. Antibodies to GAA were quantitated by ELISA as described in Materials and Methods. Data are expressed as means ± SEM (n = 5 animals per group).

GAA-treated mice displayed measurable amounts of GAA, with the cohort treated with the higher dose showing a correspondingly higher level of enzyme (Fig. 4A). Measurement of serum levels in the group treated with 5×10^{11} DRP of AAV8/DC190-GAA at 2 and 13 months postinjection showed a modest decline (~2-fold) in circulating GAA over the intervening period. Mice treated with the lower dose of virus exhibited an approximately 10-fold drop in serum levels of GAA over the same period (Fig. 4A). At 13 months posttreatment, tissue glycogen levels in mice that received the higher dose of virus were determined to be reduced to basal levels, approximating those observed in age-matched, wild-type mice (Fig. 4B). Animals that received the lower dose of AAV8/DC190-GAA showed good correction of glycogen storage in the heart and diaphragm, but the skeletal muscle showed indications of reaccumulation at the 13-month time point (Fig. 4B). This may be due to the observed progressive decline in GAA expression in this cohort as noted above. Hence, sustained high-level production of the enzyme would appear to be necessary to maintain substrate clearance over time in Pompe mice.

To evaluate the effect of glycogen clearance on motor coordination, the animals were tasked with performing on a RotaRod programmed to accelerate to increasing speeds over a period of 60 sec. As expected, in contrast to wild-type mice, untreated Pompe mice displayed a progressive loss of muscle strength and coordination beginning at approximately 6 months of age (Fig. 5A). This was indicated by the decreasing latency of the Pompe animals over the 13 months of the study. In contrast, Pompe mice treated by administration of either 10^{11} or 5×10^{11} DRP of AAV8/DC190-GAA performed with a proficiency that was indistinguishable from that of wild-type mice (Fig. 5A). Hence, AAV-mediated clearance of the majority of lysosomal glycogen from presymptomatic Pompe mice translated to significant improvements in motor function and coordination.

Beginning at 5 months of age, mice were also tested every 3 months for muscle strength by wire hang. Untreated Pompe mice displayed a loss of muscle strength as indicated by a progressively decreased latency over time (Fig. 5B). The performance of the Pompe mice treated with the higher dose of 5×10^{11} DRP of AAV8/DC190-GAA was not significantly different from that of wild-type animals. However, those that received the lower dose of virus showed some loss in muscle strength starting at the 11-month time point (Fig. 5B). This likely reflected the less than complete clearance of glycogen from the skeletal muscles of mice treated at this lower dose of AAV8/DC190-GAA, as noted above.

Treatment of older, symptomatic Pompe mice results in significant clearance of glycogen storage but only partial improvement in muscle function

To ascertain whether the encouraging data noted above also translated to older Pompe mice with more established disease manifestations, a similar study was initiated in mice

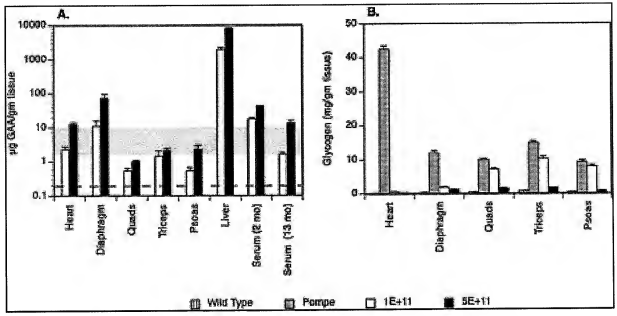


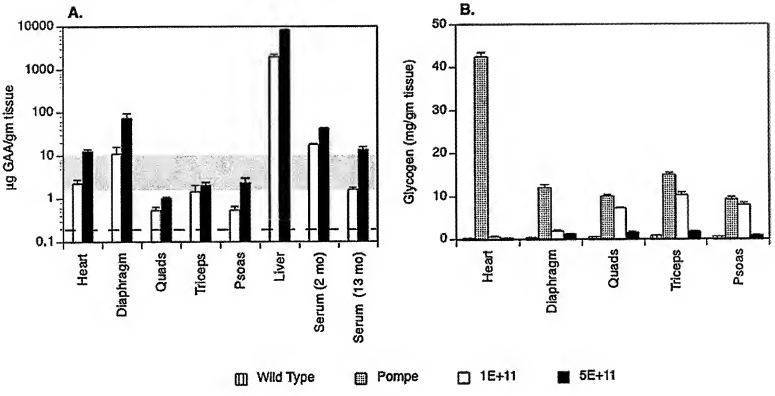
FIG. 4. Efficacy of intravenous administration of AAV8/DC190-GAA to 2-month-old Pompe mice. Groups of Pompe mice were either left untreated or administered 10^{11} or 5×10^{11} DRP of AAV8/DC190-GAA. At 13 months posttreatment, the animals were killed and their tissues were analyzed for the levels of (A) GAA and (B) glycogen. Sera were also collected by retro-orbital bleeding 2 and 13 months posttreatment and analyzed for enzyme levels. An ELISA was used to measure the levels of GAA in the various tissue homogenates. The shaded area within the graph represents the range of GAA levels observed in normal (C57BL/6) mouse tissues. The horizontal dashed line represents the limit of detection by this assay. Glycogen levels were determined by the Amplex Red glucose assay. Data are expressed as means ± SEM (n = 10 animals per group).

when they were 10 months of age (instead of 2 months). Ten-month-old Pompe mice have greater levels of lysosomal glycogen and demonstrate impaired muscle function and strength. Mice were administered 10^{12} DRP of AAV8/DC190-GAA per mouse and their tissue GAA and glycogen levels were assayed 6 months later. A higher dose of virus was used in this study because the older animals were larger and preliminary studies had indicated they were more refractory to AAV transduction (data not shown). With the exception of the quadriceps, tissue GAA levels were higher or within the range observed in wild-type mice (Fig. 6A). Measurement of serum levels 2 and 6 months posttreatment showed no significant change over this time period (Fig. 6A). At 6 months posttreatment, tissue glycogen levels in the heart and diaphragm were reduced to basal levels and those in skeletal muscles were reduced by 50 to 70% (Fig. 6B).

RotaRod analysis showed that the motor function of Pompe mice at the start of the study (10 months old) was already compromised when compared with age-matched, control wild-type mice (Fig. 7A). Treatment with AAV8/DC190-GAA resulted in significant but only partial improvement in motor coordination starting at 4 months posttreatment (Fig. 7A). However, wire hang testing showed that AAV8/DC190-GAA-treated Pompe mice exhibited only a trend toward improvement when compared with untreated animals (Fig. 7B). Hence, treating older Pompe mice resulted in significant clearance of glycogen from the affected muscles, but this did not translate into major improvements in muscle function and strength. However, it should be noted that the treated Pompe mice at 15 months of age were heavier and exhibited greater mobility and overall health than their untreated counterparts, which appeared moribund (data not shown).

Histopathological analysis of skeletal muscles of old Pompe mice shows significant myopathic abnormalities despite treatment

In an attempt to reconcile the basis for the observed difference in muscle function and strength of Pompe mice treated beginning at 2 and 10 months of age, histological sections of quadriceps were prepared and analyzed. Hematoxylin and eosin staining (Fig. 8A–D) was used to evaluate overall muscle morphology, glycogen deposits, and nuclear location. As shown in Fig. 8B, skeletal muscle isolated from 15-month-old vehicle-treated Pompe mice displayed evidence of significant glycogen accumulation (as illustrated by the white intracellular deposits). Moreover, many (25–50%) of the nuclei of the myofibers were centrally localized, suggesting the muscles had undergone a regenerative response. In contrast, nuclei of myofibers from normal mice were located primarily in the periphery (Fig. 8A). Examination of sections from Pompe mice treated at 2 months of age and analyzed 13 months later showed nearly complete clearance of the glycogen deposits and only the occasional presence of fibers with centralized nuclei (Fig. 8C). However, analysis of sections from Pompe mice treated at 10 months of age and processed 5 months later showed residual glycogen deposits and the presence of many fibers with centrally localized nuclei (Fig. 8D). Hence, the morphology of muscle cells of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Glycogen (mg/gm tissue)
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20
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Heart
Diaphragm
Quads
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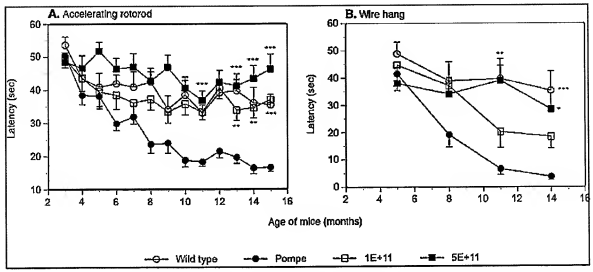


FIG. 5. Assessment of muscle function. Wild-type mice, untreated Pompe mice, and Pompe mice that were administered either 10^{11} or 5×10^{11} DRP of AAV8/DC190-GAA were subjected to (A) accelerating RotaRod and (B) wire hang tests. In the accelerating RotaRod test, the RotaRod platform was programmed to accelerate at a constant rate from 0 to 30 rpm over 60 sec. Latency to fall from the rod was recorded with a light beam sensor. Mice were tested every month posttreatment for 15 months. At each time point, every animal was subjected to three trials with a 5-min resting period between each trial. In the wire hang test, mice were placed on a wire mesh that was then inverted and their latency to fall was recorded visually with a stopwatch. Mice were tested every 2 to 3 months on the wire hang apparatus as indicated. Each animal was subjected to two trials at each time point. Data are expressed as means ± SEM ($n = 10$ animals per group). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, in comparison with the vehicle-treated Pompe group.

Pompe mice treated earlier was more akin to that of wild-type animals, whereas those treated at an older age showed histologic evidence of derangement.

Sections of quadriceps were also stained with trichrome blue for evidence of fibrosis (Fig. 8E–H). Vehicle-treated 15-month-old Pompe mice showed the presence of thickened endomysium throughout the sections (Fig. 8F). Inflammatory cell infiltrates were also evident in some areas. These findings indicate mild fibrosis and shrinking of the myofibers. Early intervention with AAV8/DC190-GAA prevented the formation of these pathological lesions (Fig. 8G) whereas those treated later looked similar to untreated controls (Fig. 8H). It is possible that these lesions were already present in the muscles of the 10-month-old Pompe mice and that treatment with AAV8/DC190-GAA was only partially effective in abating or reversing this manifestation. These data suggest that it may be more difficult to rescue muscle pathology and function after the onset of significant disease. Moreover, there was a correlation between decreased motor function and the presence of more extensive pathology.

Discussion

Clinical studies have shown that biweekly infusions of recombinant GAA to infantile subjects with Pompe disease result in reversal of the hypertrophic cardiomyopathy, improvement in skeletal muscle function, and importantly, increased survival (Kishnani *et al.*, 2007). These positive findings led to the approval of enzyme replacement therapy for treating patients with Pompe disease. However, it was noted that correction of the glycogen storage in the heart was more effective than in skeletal muscle. In contrast to the observation of marked improvement in cardiomyopathy in all the treated patients, improvement in motor function was noted only in a proportion of the treated subjects. Preclinical studies in Pompe mice had suggested that this discordance might be due to less efficient delivery of GAA to the skeletal muscle. The disparity in efficiency of delivery to the two organs may be related to the presence of lower levels of the cation-independent mannose 6-phosphate receptor, which is primarily responsible for cellular uptake of the enzyme, on skeletal muscle (Wenk *et al.*, 1991). Access of proteins from the systemic circulation to the interstitia of cardiac muscle is also apparently greater compared with skeletal muscle (Renkin *et al.*, 1989). Although this issue could potentially be addressed by administering greater quantities of the enzyme, such a strategy could also exacerbate the host immune response, which in turn may impact efficacy, particularly in cross-reactive immunological material (CRIM)-negative Pompe subjects (Raben *et al.*, 2003; Cresawn *et al.*, 2005; Kishnani *et al.*, 2006, 2007). Other aspects of enzyme therapy that may present a challenge include the short half-life of GAA (van der Ploeg *et al.*, 1991) and the ability of this approach to address the apparently dysfunctional autophagocytic activity observed in Pompe cells (Fukuda *et al.*, 2006; Raben *et al.*, 2007).

Reports suggest that gene augmentation therapy may represent an alternative and potentially improved approach to deliver GAA systemically (Fraites *et al.*, 2002; Mah *et al.*, 2005, 2007; Sun *et al.*, 2005, 2006, 2007). For example, sustained therapeutic levels of the enzyme could be realized after a single administration of recombinant AAV vectors encoding

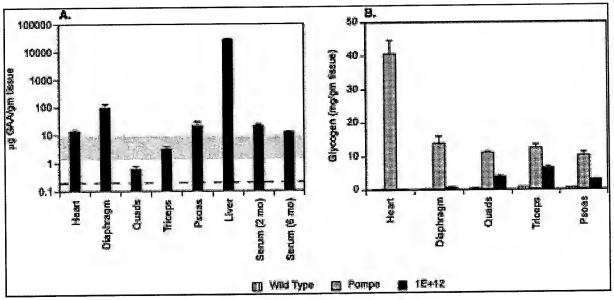
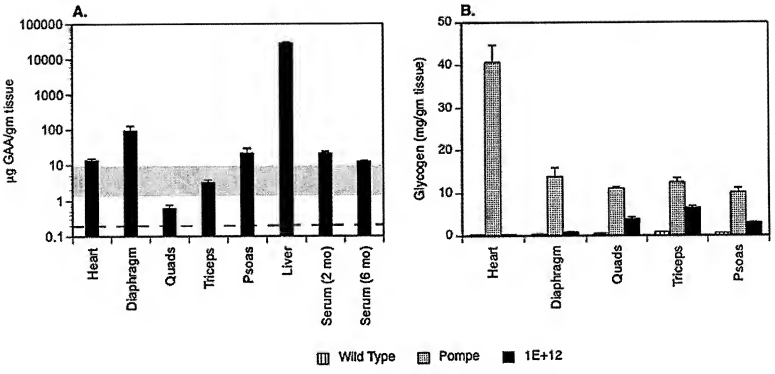


FIG. 6. Efficacy of intravenous administration of AAV8/DC190-GAA to 10-month-old Pompe mice. Groups of Pompe mice were either left untreated or administered 10^{12} DRP of AAV8/DC190-GAA. At 6 months posttreatment, the animals were killed and their tissues were analyzed for the levels of (A) GAA and (B) glycogen. Sera were also collected by retro-orbital bleeding at 2 and 6 months posttreatment and were analyzed for enzyme levels. An ELISA was used to measure the levels of GAA in the various tissue homogenates. The shaded area within the graph represents the range of GAA levels observed in normal (C57BL/6) mouse tissues. Glycogen levels were determined by the Amplex Red glucose assay. Data are expressed as means ± SEM (n = 7 animals per group).

GAA, which translated to significant reductions in tissue glycogen levels. Genetic modification of the liver or muscle to synthesize and secrete the enzyme into the systemic circulation was efficacious, although results with liver as the depot organ were more effective. Our results, using a recombinant AAV8 serotype vector, confirm these reports. Administration of a high dose of AAV8/DC190-GAA to young presymptomatic Pompe mice led to rapid and nearly complete correction of the glycogen storage abnormality in all the affected muscles. Moreover, clearance of tissue glycogen was correlated with significant improvements in muscle function. These responses were dose dependent, with animals treated at higher doses generating correspondingly higher levels of GAA and concomitantly greater improvements in motor function. At the highest dose tested, muscle strength and coordination were indistinguishable from those of age-matched wild-type mice. These observations suggest that early intervention with gene therapy can effectively abate the onset and progression of Pompe disease.

Pompe mice exhibit pathology in the CNS (data not shown) that arguably could have impacted their performance in the functional tests. Although it has been suggested that administration of high doses of other lysosomal enzymes, such as β-glucuronidase (Vogler *et al.*, 2005) and arylsulfatase A (Matzner *et al.*, 2005), could allow for some to traverse the blood–brain barrier, it is unlikely that this occurred in the Pompe mice described here, or could have accounted for the observed improvement in motor function. Analysis of brains from Pompe mice treated at the highest viral dose showed only marginal reductions in glycogen levels (data not shown). Moreover, results following systemic delivery of an AAV vector encoding acid sphingomyelinase into Niemann-Pick A mice (another lysosomal storage disease) demonstrated that this did not improve motor coordination or cognition (Passini *et al.*, 2007). Benefits were realized only when the AAV vector was introduced directly into the brain of these animals.

The observed requirement for high, supraphysiological levels of GAA for positive outcome in Pompe mice contrasts with that for other lysosomal storage diseases, where typically reconstitution of the respective enzymes to ~50% of normal levels would appear to be therapeutic. In part, this may be attributed to the increased challenge associated with delivering GAA from the systemic circulation across the various physiological barriers to the affected muscle cells. It has also been suggested that intracellular trafficking of the lysosomal enzyme and glycogen to the lysosomal compartment may be deranged in the diseased skeletal muscle, which would lessen the efficacy of treatment (Raben *et al.*, 2002). Studies have also highlighted the differential response of different Pompe muscle fiber types to enzyme therapy, with the slow twitch-predominant muscles (type I) being more responsive to treatment than their fast twitch-predominant (type II) counterparts (Raben *et al.*, 2002; Hawes *et al.*, 2007). A correlate was observed between high capillary density (which is normally associated with slow twitch-predominant muscles) and greater glycogen clearance, suggesting that the differential bioavailability of GAA to the different muscle types may be responsible for the observed disparity in glycogen clearance. If correct, the use of gene therapy, by virtue of its ability to facilitate continuous production of high levels of GAA into the systemic circulation, thereby enhancing


A.
μg GAA/gm tissue
100000
10000
1000
100
10
1
0.1
Heart
Diaphragm
Quads
Triceps
Psoas
Liver
Serum (2 mo)
Serum (6 mo)
B.
Glycogen (mg/gm tissue)
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Wild Type
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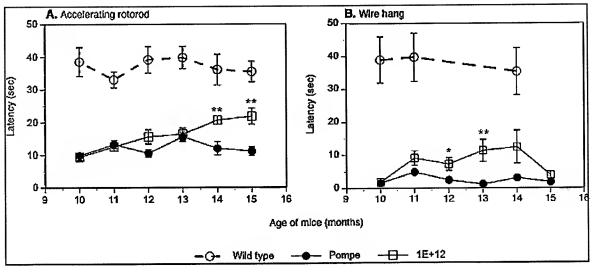

FIG. 7. Assessment of motor function and strength. Wild-type mice, untreated Pompe mice, and Pompe mice that were administered 10^{12} DRP of AAV8/DC190-GAA were subjected to (A) accelerating RotaRod and (B) wire hang tests. In the accelerating RotaRod test, the RotaRod platform was programmed to accelerate at a constant rate from 0 to 30 rpm over 60 sec. Latency to fall from the rod was recorded with a light beam sensor. Mice were tested every month posttreatment for 5 months. At each time point, every animal was subjected to three trials with a 5-min resting period between each trial. In the wire hang test, mice were placed on a wire mesh that was then inverted and their latency to fall was recorded visually with a stopwatch. Mice were tested every month posttreatment on the wire hang apparatus. Each animal was subjected to two trials at each time point. Data are expressed as means ± SEM ($n = 7$ animals per group). *$p < 0.05$ and **$p < 0.01$ in comparison with the vehicle-treated Pompe group.

its bioavailability, could represent an improvement over intermittent administration of recombinant enzyme in overcoming these challenges.

Interestingly, although treatment of older, symptomatic Pompe mice with the recombinant AAV vector resulted in a significant reduction (albeit incomplete) in tissue glycogen levels, this translated into only a modest improvement in skeletal muscle function. Hence, unlike the observation in younger Pompe mice, reducing glycogen levels per se in the older and chronically diseased muscle was not correlated with a similar robust improvement in muscle function. This finding suggests that other aspects of muscle physiology might have been impacted by the disease process in the older animals that could not be rescued by merely removing the offending agent. Indeed, it was noted that there was evidence of mild fibrosis in the skeletal muscle of the older animals. However, it is possible that in our studies we had not allowed sufficient time for muscle function to recover and that an extended recovery period (beyond the 6-month test period) may have revealed more substantial improvements.

The basis for the inability to completely clear tissue glycogen (despite attaining high tissue enzyme levels) from the skeletal muscles of older mice is unclear. It may be due to greater accumulation of the substrate in the older animals or perhaps to changes in the properties of the stored glycogen such that they were rendered more resistant to digestion. Alternatively, the milieu within the lysosomal compartment may have been altered by disease to be less compatible for digestive action. Yet another and perhaps more likely consideration is the observation of disrupted trafficking of glycogen-laden autophagosomes to the lysosomes in old Pompe mice (Raben *et al.*, 2002, 2007). This purportedly can lead to subsequent enlargement of the glycogen-filled autophagic vesicles in a proportion of the affected cells. The progressive intracellular accumulation of these large autophagic vesicles has been suggested to be a contributory factor to skeletal muscle damage. Examination of histologic sections indicated evidence of structural pathology in the skeletal muscle that may have contributed to the poor response in motor function. These pathological findings were not observed in the tissues of mice treated at 2 months of age, suggesting that early therapeutic intervention was important in preventing the onset and development of these traits. The importance of early treatment has also been noted in clinical studies, in which a more robust outcome was observed in infantile Pompe subjects treated before they were 6 months old than in those treated between 6 and 36 months of age (Kishnani *et al.*, 2006, 2007). The preclinical data presented here confirm that ideally, treatment should be administered before the development of significant disease pathology and is supportive of early therapeutic intervention in managing Pompe disease.

The ability of AAV vectors harboring liver-restricted promoters to facilitate the development of immunotolerance to the expressed transgene product, including the lysosomal enzymes α-galactosidase A, acid sphingomyelinase, and acid β-glucosidase, has been demonstrated previously (Mingozzi *et al.*, 2003; Ziegler *et al.*, 2004; Barbon *et al.*, 2005; McEachern *et al.*, 2006). Our findings here suggest that the same end result could be attained for GAA, although much higher levels of expression of this enzyme were required than were needed for the other lysosomal hydrolases. Administration

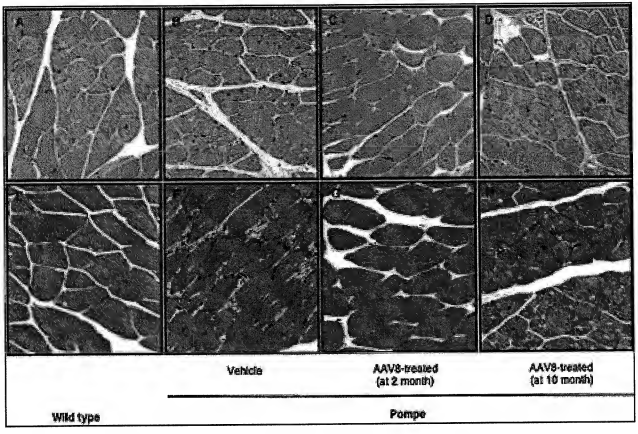

FIG. 8. Histopathological analysis of skeletal muscles of Pompe mice. Representative sections of quadriceps from 15-month-old wild-type mice (**A** and **E**), 15-month-old untreated Pompe mice (**B** and **F**), 15-month-old Pompe mice treated with AAV8/DC190-GAA at 2 months of age (**C** and **G**), and 15-month-old Pompe mice treated with AAV8/DC190-GAA at 10 months of age (**D** and **H**) were either stained with hematoxylin and eosin (**A–D**) or trichrome blue (**E–H**). Original magnification, ×40.

of less than 10^{11} DRP of AAV8/DC190-GAA per mouse or production of less than 1 μg of GAA per milliliter into the serum was ineffective at inducing immunotolerance. The basis for the requirement for higher levels of GAA is unclear but may be related to the intrinsic antigenicity of the protein. Nevertheless, the ability to induce tolerance by gene therapy may support consideration for the use of this technology to treat CRIM-negative infantile Pompe patients, as suggested by Sun and coworkers (2007). It is estimated that approximately 3 to 5% of infantile Pompe patients are CRIM negative. These infants are at high risk of developing persistent antibodies after enzyme therapy; in a minority of cases, antibodies had inhibitory *in vitro* activity (Kishnani *et al.*, 2006, 2007). The ability to tolerize these subjects by gene transfer before enzyme therapy (if necessary) represents one avenue by which this limitation may be overcome.

It is evident that systemic gene therapy using recombinant AAV8 vectors can address some of the limitations shown to be associated with enzyme replacement therapy for Pompe disease. Moreover, it is apparent that initiation of therapy before the onset of significant disease pathology, particularly in skeletal muscle, is an important consideration for treating this disease. The immunotolerization data reported here also support the potential application of this approach to treat CRIM-negative infantile Pompe subjects. However, translating this technology into the clinic will require a better understanding of the role of preexisting immunity to the viral vectors (Mingozzi *et al.*, 2007). It will also require exploration of AAV vector manufacturing at sufficient scale. In this regard, developments in AAV manufacturing and in the use of immunosuppressants as an adjunctive therapy are encouraging (Jiang *et al.*, 2006; Mingozzi and High, 2007). Thus, renewed interest in further testing this promising technology for clinical use is warranted.

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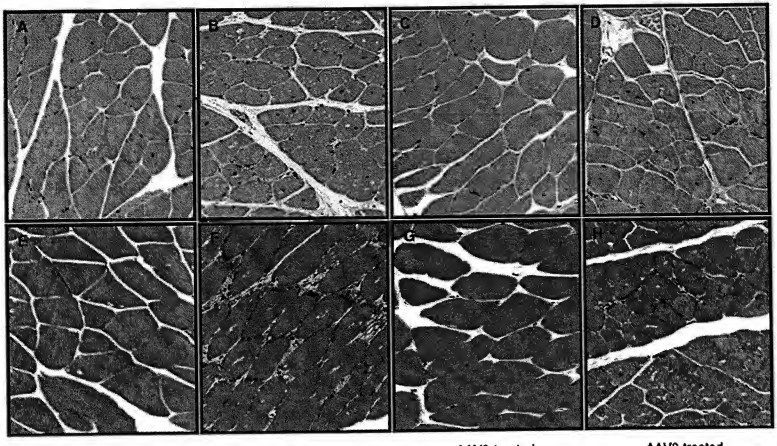
Author Disclosure Statement

All authors are employees of Genzyme Corporation.



Vehicle

AAV8-treated (at 2 month)

AAV8-treated (at 10 month)

Wild type

Pompe

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Address reprint requests to:
Dr. Seng H. Cheng
Genzyme Corporation
49 New York Avenue
Framingham, MA 01701-9322

E-mail: seng.cheng@genzyme.com

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